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**EFFECT OF JACKFRUIT-DERIVED EXTRACT CONSUMPTION ON
COLITIS-ASSOCIATED COLON TUMORIGENESIS IN MICE**

A Thesis Presented

by

JINGWEN LIN

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2020

Food Science

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ABSTRACT

EFFECT OF JACKFRUIT-DERIVED EXTRACT CONSUMPTION ON COLITIS-ASSOCIATED COLON TUMORIGENESIS IN MICE

SEPTEMBER 2020

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Colorectal cancer is the third most common cancer and the fourth most common cause of cancer-related death in the world. The global burden of colorectal cancer is also expected to increase by 60%, to over 2.2 million new cases and 1.1 million annual deaths, by the year 2030. Jackfruit is known for its packed nutrition including many antioxidants: vitamin C, carotenoids and flavanones. It has also been used in traditional medicine due to its potential protection against many chronic diseases. However, there is limited research studying the potential effect of jackfruit on colorectal cancer. Here, we used a well-established AOM/DSS mice model to investigate the impact of jackfruit-derived extracts on colitis-associated colorectal cancer. After 6-week treatment with diet containing 480 ppm jackfruit-derived extracts, the mice showed significantly alleviated colon tumorigenesis with a 46% decrease in tumor numbers of each mouse compared to vehicle group (2.1 ± 0.31 for 480 ppm jackfruit-derived fraction group vs 3.9 ± 0.67 for vehicle group, $P < 0.05$). The expression of the pro-inflammatory cytokines (*Il-6* and *Inf- γ*) and pro-tumorigenic genes (*Axin2*, *Vegf*, *Myc* and *Pcna*) was also decreased in the group consuming 480 ppm jackfruit-derived extracts compared to the vehicle group. Together the results suggest that the consumption of jackfruit-derived extracts could protect against colitis-associated colorectal carcinogenesis in mice.

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction of Human Colorectal Cancer

1.1.1 Incidence

Colorectal cancer is the third most commonly diagnosed form of cancer globally, comprising 10.2% of all cancer diagnoses in 2018, according to WHO [1], [2].

Colorectal cancer is also the third most common cancer in the United States, according to American Cancer Society. It is estimated that there will be 147,950 new cases of colorectal cancer in the U.S in 2020. Overall, the lifetime risk of developing colorectal cancer in the U.S. is about 1 in 23 (4.4%) for men and 1 in 25 (4.1%) for women [3]. The incidence rate escalates rapidly with age in the U.S., approximately doubling with each 5-year age increase until age 50 years and increasing by approximately 30% with subsequent groups aged 55 years and older [4].

1.1.2 Mortality

Colorectal cancer is the second most deadly cancer worldwide, with about 881,000 deaths estimated for 2018. The cumulative risk, at age 0 to 74 years, of dying from colorectal cancer is 1.12% among men and 0.70% among women globally [1]. In the United States, colorectal cancer is the third leading cause of cancer-related deaths in men and in women, and the second most common cause of cancer deaths when men and women are combined. The percent of colorectal cancer deaths is highest among people aged 75–84, with a median age at death 74 [5]. It's expected to cause about 53,200 deaths in the U.S. during 2020 [3].

1.1.3 Trend

The global burden of colorectal cancer is expected to increase by 60%, to over 2.2 million new cases and 1.1 million annual deaths, by the year 2030 [6]. In the United States, the incidence rates of colorectal cancer for ages 20–49 years was 9.3 per 100,000 in 1975 and is up to 13.7 per 100,000 in 2015, a percentage increase of 47.31%, although incidence rates in age groups 50 years and above has steadily decreased until 2011 [7]. Among individuals aged 50 to 64 years, however, declines in incidence of 2% to 3% per year during the 2000s have reversed in recent years, with rates during 2011 through 2016 increasing by 1.0% per year [8]. Over the past 10 data years (2008-2017) in the United States, mortality rate increased by 1.3% per year in individuals younger than 50 years, though death rates declined by 3% per year in individuals aged 65 years and older and by 0.6% per year in individuals aged 50 to 64 years [9].

1.2 Animal Models of Colorectal Cancer

With the large incidence and mortality of colorectal cancer, furthering scientific understanding of the disease to promote more effective preventions, diagnoses, and treatments presents an important and challenging problem. Controlled in vivo animal studies are one of the primary ways for the scientific community to conduct ethical studies in which the tumorigenesis process can be carefully studied in a biological setting approximating the human colon. There are a few aspects involved in selecting a good animal model for colorectal cancer study. First, the cancer that develops in the animal model should be limited to the large intestine or rectum so that researchers can study the development of the disease without the confounding effects of disease in other tissues. Second, the histologic and molecular features of colorectal lesions should be similar to

those observed in human tissue. Finally, the models should capture the complex cellular interactions that are relevant to human colon cancer. For example, though xenografts of human tumor into nude mice are often cited as highly relevant to the study of human cancer, these mice are immune-compromised and this eliminates the impact of immune system on the tumors [10]. Due to the similarities of genetic and physiological traits mice possess to those of humans, and relatively low maintenance requirements compared to larger mammals (e.g. pigs or dogs), mice are better models for studying the immune, endocrine, nervous, and other physiological systems. Like humans, mice have the ability to develop several diseases, such as cancer [11]. For research purposes, two major animal models of colorectal carcinogenesis are chemical or environmental agents induced colorectal tumors in rodents, which represent sporadic colorectal cancer, and genetically modified mice, which represent the hereditary familial adenomatous polyposis and hereditary non-polyposis colorectal cancer syndromes (HNPCC) [12].

1.2.1 Colorectal cancer induced by exogenous agents

A large number of chemicals are known to have mutagenic potential, and chemically induced tumors on rodents are generally used to study the wide range of epigenetic alterations found in sporadic or colitis-associated colorectal cancer [13]. Administration of the chemical compounds is possible via ad libitum feeding, oral gavage, intraperitoneal or intramuscular injection, or enema [14].

1.2.1.1 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM)

DMH and its metabolite AOM are the two most commonly used carcinogens to induce and promote colorectal cancer in rats and mice [15]. DMH is metabolically activated in the liver by a series of reactions through intermediates AOM and

methylazoxymethanol (MAM) which are metabolized predominantly by CYP2E1 in the liver and other enzymes in extrahepatic organs to the ultimate carcinogenic metabolite — highly reactive methyldiazonium ion — causing alkylation of DNA bases [16], [17]. DMH and AOM are alkylating agents that are typically injected intraperitoneally or subcutaneously over several weeks to induce development of tumors in the distal colon. The majority of these tumors harbor mutations in the β -catenin gene (*Ctnnb1*, *the Ctnnb1 gene encodes β -catenin*), which is similarly observed in hereditary nonpolyposis colorectal cancer in humans [18]. These mutations affect the N-terminal amino acids of the β -catenin gene product, making the protein resistant to regulatory degradation, stabilizing β -catenin, and increasing WNT signaling to drive tumorigenesis [19]. In contrast to the majority of genetic mouse models producing tumors mainly in the small intestine, DMH/AOM-treated mice generate tumors predominantly in the distal colon, which enable researchers to study pathological process of colorectal cancer without the confounding factors of diseases in other tissues. In addition, tumor incidence and multiplicity can be altered by both genetic background and by diet [15]. This makes the models advantageous for the study of gene-gene and gene-environment interactions that influence the pathogenesis of colorectal cancer. However, some have questioned the translational potential of data generated with DMH/AOM model, since there is little evidence that a large proportion of human sporadic colorectal cancer results from exposure to alkylating agents [10]. As mentioned before, the activation of DMH/AOM carcinogenesis relies on the participation of various enzymes in the liver, therefore chemopreventative agents with potential interference with the metabolic pathway of DMH/AOM may modify colon carcinogenesis and affect study results [16], [17]. To

avoid interference between the compound and DMH/AOM activation, chemopreventive treatment can begin after carcinogen administration, that is, during the promotion or progression phase [20].

1.2.1.2 AOM / Dextran sulfate sodium (DSS)

DSS-induced colitis animal model is widely used because of its simplicity and many similarities with human ulcerative colitis. The mechanism by which DSS induces intestinal inflammation is unclear but it is likely the result of damage to intestinal epithelial monolayer lining, leading to the entry of luminal bacteria and associated antigens into the mucosa and allowing the dissemination of proinflammatory intestinal contents into underlying tissues [21]. To create a model of colorectal tumors associated with chronic inflammation, a protocol combining AOM with an inflammatory agent, dextran sulfate sodium (DSS) salt, was introduced by Suzuki *et al* in 2007 [22]. Chronic inflammation leads to the formation of a microenvironment enriched with immune cells that produce pro-inflammatory cytokines and growth factors and, simultaneously, increase the local levels of reactive oxygen species. Subsequently, cell proliferation and the risk of DNA damage are increased. In the case of a long-lasting inflammatory response, cell transformation and tumorigenesis occur with high frequency. One of the evident advantages of the AOM and DSS combination is further reduction in the time needed for tumor formation. A single dose of AOM followed by five days of DSS treatment resulted in development of multiple colon tumors within 10 weeks compared to 24-50 weeks of latency when only DMH/AOM is used [23], [24]. More importantly, studies in mice and rats have revealed that AOM/DSS-induced tumors display very similar features to human CRC even at the molecular level. Inactivating APC mutations

or activating β -catenin mutations, which mimics WNT stimulation and leads to β -catenin accumulation, is observed in the majority of colon cancers in both humans and rodents [25], [26]. Also consistent with findings in human colorectal cancer, AOM/DSS-induced tumors also have mutations of K-Ras and increased levels of enzymes involved in prostaglandin and nitric oxide synthesis, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [27], [28]. However, a recent study that performed exome sequencing on colorectal cancer mice under AOM/DSS treatment and humans with colorectal cancer found that the top 20 most frequent mutation sites including APC, TP53, KRAS, NRAS, BRAF, PIK3CA, SMAD4 and FBXW7 in human CRC samples were not detected in AOM/DSS mice, questioning the compatibility of using AOM/DSS mouse model to mimic human colorectal cancer [29]. Nonetheless, because of its high reproducibility and potency, as well as the simple and affordable mode of application, the AOM/DSS model has become an outstanding model for studying colon carcinogenesis and a powerful platform for chemopreventive intervention studies [30].

1.2.1.3 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP)

PhIP is the most abundant heterocyclic aromatic amines (HAA) formed from the reaction between free amino acids, sugars and creatine at high temperatures during the cooking process of meat and fish, which most people are exposed to in daily life [31]. PhIP is metabolized by the liver enzyme CYP1A2 to N2-Hydroxy-PhIP, which then, after sulfation or acetylation, forms activated esters capable of DNA adduct formation [32], and then induces formation of colonic aberrant crypt foci, but fails to induce formation of colon tumors in mice [33], [34]. However, combining PhIP with either DSS treatment or treating *Apc^{Min}* mice with PhIP can enhance tumorigenesis [35], [36]. Data obtained from

the study of PhIP in rodents is considered to be highly relevant to human cancer, since epidemiologic evidence links PhIP from cooked meat to increased colorectal cancer risk [37], [38]. In addition, stimulation of colon tumorigenesis by PhIP also occurs in mice consuming a high-fat diet, however the tumor incidence was relatively low [39], [40], [41]. PhIP was also found to lead to formation of mammary and prostate neoplasia besides causing colon tumorigenesis [42], which can lead to confounding effects in research.

1.2.1.4 N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (abbreviated as both MNU and NMU)

MNNG and MNU are direct-acting carcinogens which have been administered to mice and rats to induce neoplasia in a variety of organs [43]. They are carcinogens which methylate nucleic acids and proteins, and covalently modify proteins [44]. However, some studies have found that MNU has failed to induce tumorigenesis selectively in colorectal tissue when administered orally, injected intraperitoneally or intramuscularly, and intrarectally, indicating that MNU might be a less competent carcinogenesis agent when studying the pathological progress of just colorectal cancer [45], [46], [47], [48]. On the other hand, it has been discovered that when MNNG was given intrarectally at a dose rate of 1–3 mg/rat/week for 20 weeks, it induced colon tumors in 100% of male F344 rats, and the neoplasms were all located in the distal colon and rectum [49]. However, the major weakness of MNNG is that the technique of intrarectal injection requires highly skilled technicians and quantification of carcinogens instilled intrarectally is difficult [50].

1.2.2 Genetically modified mice models

The development of gene targeting has enabled researchers to have a more controlled approach when studying colorectal cancer. The availability of genetic and genomic information, the ease of genetic manipulation through mutagenesis techniques and the ability to monitor the effects on a whole organism have made genetically engineered mice a very advantageous model of CRC. These advantages make mouse models fundamental when testing therapeutics, enabling efficacy and toxicity of the treatments to be analyzed but have their own limitations which will also be discussed here.

1.2.2.1 Mice models for FAP

FAP is an autosomal-dominant colorectal cancer syndrome, caused by a germline mutation in the adenomatous polyposis coli (APC) gene, on chromosome 5q21. It is characterized by hundreds of adenomatous colorectal polyps, with an almost inevitable progression to colorectal cancer with a high risk of metastasis at an average age of 35 to 40 years [51]. One of the most widely used animal model for FAP is a type of mice termed Min or *Apc*^{min/+} (multiple intestinal neoplasms). This mouse model was obtained by random mutagenesis using *N*-ethyl-*N*-nitrosourea. The Min mutation was found to have an autosomal dominant mutation at codon 850 in APC causing a truncated protein of 850 amino acids [52]. *Apc*^{min/+} heterozygotes are born normally and have a reduced average lifespan of 150 days. These mice can develop more than 100 adenomas in the small intestine, and a small number of polyps in the colon depending on the genetic background [53]. *Apc*^{min/+} mouse model has phenotypic and genetic similarities to FAP patients, making it an ideal model to investigate colon cancer. However, this model does not completely match the phenotype of FAP, an example being that FAP patients develop

adenomas predominately in the colon, whereas adenomas in *Apc^{min/+}* mice are predominantly localized to the small intestine. One of the solutions is to treat *Apc^{min/+}* female mice with 2% DSS, as Tanaka *et al.* and Cooper *et al.* have reported the increase in the incidence of colonic neoplasms and dysplastic crypts in female *Apc^{min/+}* mice administered with DSS [54], [55]. Another solution is to cross *Apc^{+/loxP}* mice with mice carrying *CDX2P 9.5-NLS Cre* transgene. Hinoi *et al.* reported that *CDX2P-NLS Cre;Apc^{+/loxP} (CPC;Apc)* mice were found to develop 5 to 8 tumors in the colon and rectum, and only 3 tumors in distal small intestine on average [56]. Another limitation of the *Apc^{min/+}* mouse model is that the adenomas are generally benign and do not progress to invasive colon cancer, probably due to the short lifespan of the mice.

FAP patients have been found to exhibit a wide variety of APC mutations, which can affect both the phenotype and the prognosis [57]. To understand the precise roles of different APC mutations on the development of colorectal cancer, further studies were conducted using mice that carry different APC mutations which have been implicated in either the initiation or progression of colorectal cancer. These mouse models containing different mutations in the APC gene differ phenotypically by the number of polyps in the small intestine, colon, and levels of invasion. For example, a heterozygous mouse model for a truncation mutation at codon 716 in APC (*Apc^{Δ716/+}*) contain 10 times the number of polyps in the small intestine compared to the *Apc^{min/+}* mouse model [58], whereas the insertion of a neomycin cassette into exon 15 (*Apc^{I638N}*) results in fewer polyps but with a marked increase in invasion [59]. Research in heterozygous APC mutations has not only increased the range of animal research models available for study of the disease, it also led to insights into the relationships between APC mutation type and human patient

prognosis. An example of this is the mouse model heterozygous for a truncation mutation in the APC gene at codon 1309 (*Apc*^{I309/+}). This mouse model has a more severe phenotype than the *Apc*^{min/+} mouse model, including a shorter lifespan and an increase in polyps in the colon, and FAP patients with this mutation also have an earlier onset of the disease and severe polyposis, making their phenotype more severe than patients with other APC mutations [60].

1.2.2.2 Mice models for HNPCC

HNPCC is the most frequent form of hereditary colorectal cancer, and accounts for up to 5% of all colorectal cancers in the United States. HNPCC is inherited in an autosomal dominant fashion with high penetrance. Colorectal cancer patients diagnosed with HNPCC suffer an early onset of tumorigenesis, with a small subset of patients also developing tumors in the stomach and small intestine [53]. HNPCC is caused by mutations in the mismatch repair genes including MLH1, MSH2 and MSH6, which causes microsatellite instability, characterized by increased rates of replication errors [61]. In order to better understand this disorder, a mouse model that closely mirrors the genetic scenario in humans via heterozygous deletion of these genes was developed. However, this mouse model was unable to develop early-onset tumors, probably due to the short lifespan of mice [62]. On the other hand, homozygous knockout mice are cancer prone and develop tumors in multiple organs, and although they do develop gastrointestinal tumors, the cause of death is aggressive lymphoma [63], which does not yield an accurate model for HNPCC. By combining homozygous mutations for the mismatch repair genes with various germ-line APC mutations, much more accurate

models of HNPCC have been developed which display a phenotype of multiple early-onset intestinal tumors [64], [65], [66].

1.3 Dietary factors in colorectal cancer

1.3.1 Dietary factors in human colorectal cancer

A higher incidence of colorectal cancer is observed in North America and Europe, whereas Africa and Asia have a lower incidence. In addition to the impact of race on the risk of colorectal cancer, a variety of epidemiological studies provide compelling evidence that diet and nutrition are important factors in the development of colorectal cancer in humans. During the past three decades, many large epidemiologic studies led by many prominent research institutes have discovered interesting associations between different diet choices and the risk of colorectal cancer.

Some of the most prominent epidemiological studies on diet and colorectal cancer are the NIH-AARP DHS study (291,988 men and 197,623 women aged 50-71 years at baseline in 1995-1996 during 5 years of follow-up) [67], the EPIC study (>500,000 participants from 10 European countries) [68], and the Scandinavian HELGA study (108,000 Danish, Swedish, and Norwegian people) [69]. Multiple studies including the DHS study discovered a positive association of red/processed meat with the risk of colorectal cancer [70], [71], [72]. Accordingly, the World Cancer Research Fund and the American Institute for Cancer Research listed red/processed meat as convincing factors for increasing the risk of colorectal cancer [73], [74]. High fiber intake from whole grains and cereals was found to be associated with a lower risk of colorectal cancer, supported by the EPIC study (cereals: Relative Risk: 0.87, 95% CI: 0.77–0.99, p -trend =0.003) [68], the DHS study (grain: Relative Risk: 0.51, 95% CI: 0.29–0.89, p -trend =0.01) [67], and

the Scandinavian HELGA study (whole-grain wheat: Incidence Rate Ratio: 0.65, 95% CI: 0.50–0.84) [69]. Fruit and vegetable intake is also associated with the risk of colorectal cancer: the EPIC study observed a lower risk of colorectal cancer with higher consumption of fruits and vegetables combined among never and former smokers (Hazards Ratio: 0.86, 95% CI: 0.75–1.00, p -trend =0.04), while interestingly the consumption of fruits and vegetable was positively associated with colorectal cancer in current smokers [68]. A pooled meta-analysis by Wu *et al.* in 2012 that focused only on cruciferous vegetables and included 24 case–control and 11 prospective studies found a significant inverse relationship (Relative Risk: 0.82, 95% CI: 0.75–0.90) between cruciferous vegetable intake and the risk of colorectal cancer [75].

In addition to showing correlations between colorectal cancer risk and consumption of specific foods, epidemiological studies have also shown insightful results about the potential effects of overall, population-level diet styles on colorectal cancer. Researchers have observed that descendants of Japanese immigrants living in Hawaii were less likely to eat “Japanese-style” meals and more likely to eat “western-style” meals than first generation Japanese immigrants. In addition, US-born Japanese, regardless of sex, were taller, weighed more, and reported consuming higher quantities of meat, coffee, and total fat, but ate less tofu, green tea, and carbohydrates than Japanese living in Japan [76]. A classic 1999 study by Flood *et al.* found that US-born Japanese men experienced incidence rates of colorectal cancer twice as high as foreign-born Japanese men and about 60% higher than those of US-born white men [77]. Assuming Japanese Americans have a relatively similar genetic profile compared to Japanese living in Japan, these results, in

combination with the dietary patterns of US-born Japanese noted above, suggest that the lifestyle of a “western-style” diet may substantially increase the risk of colorectal cancer.

1.3.2 Dietary factors in animal colorectal cancer models

Besides the support of epidemiological studies on the strong connections between food/diet and the development of human colorectal cancer, many animal (especially mouse/rat) colorectal cancer studies have also shown the impact of food/diet on the risk of developing colorectal cancer. Fisher 344 rats administered with the carcinogen DMH were utilized to test the effect of red meat and calcium on colorectal cancer development. In comparison of the control rats, increased aberrant crypt foci (ACF) and mucin-depleted foci (MDF) were observed in rats whose diet contains 60% red meat, while the effect was inhibited by the addition of calcium [78]. Fernández *et al.* discovered that a prebiotic-inulin reduced colon polyps caused by the over consumption of traditional red/processed meat by 49% in Fisher 344 rats administered with AOM/DSS [79]. Donohoe *et al.* found the combination of high-fiber diet and a probiotic-*Butyrivibrio fibrosolvens* protected BALB/c mice from AOM/DSS induced colorectal cancer [80]. Sulforaphane, a component of cruciferous vegetables, appears to be an effective anti-cancer agent in cell culture, carcinogen-induced, and genetic cancer models. When sulforaphane is added at 300 or 600 µg/g to an AIN-76A diet for *Apc*^{Min} mice, it reduced the average number of polyps significantly (25.3% and 47% respectively in the small intestine), and no polyps were observed in the large intestine [81]. In sum, multiple animal models have been utilized in aiding the characterization of food and diet on the risk of colorectal cancer development.

CHAPTER 2

EFFECT OF THE CONSUMPTION OF JACKFRUIT-DERIVED FRACTION ON COLON TUMORIGENESIS IN MICE

2.1 Introduction

Artocarpus heterophyllus, which is commonly known as jackfruit or jackfruit tree is a tropical climacteric fruit, belonging to the fig, mulberry, and breadfruit family (Moraceae) [82], is native to Western Ghats of India and common in Asia, Africa, and some regions in South America [83]. It is known to be the largest edible fruit in the world [84]. Jackfruit is rich in nutrients including carbohydrates, proteins, vitamins, minerals, and phytochemicals including lignans, isoflavones and saponins that led to its extensive use in traditional medicine due to its anticarcinogenic, antimicrobial, antifungal, anti-inflammatory, wound healing, and hypoglycemic effects [85]. In addition, Ruiz-Montanez *et al.* observed that jackfruit possesses compounds with chemoprotective properties to reduce the mutagenicity of aflatoxin B1 (AFB1) and proliferation of cancer cells [86]. Chen *et al.* also discovered that artocarpin, a compound extracted from jackfruit tree, exhibited potent cytotoxicity against human colon cancer cells including DLD1, HCT15, HCT116, HT29, and SW480 cells with IC50 values at around 15 $\mu\text{mol/L}$. The attenuation of colorectal tumorigenesis was also shown in AOM/DSS mice administered with 100 mg/kg artocarpin by oral gavage, with a significant increase of mice survival rate and reduced multiplicity of colon neoplasms by 56% ($P < 0.001$) [87]. Meanwhile, water-soluble polysaccharides extracted from jackfruit tree failed to show strong toxicity to human colon tumor cells, despite its exhibited immunomodulatory

activity as well as significant anti-oxidative effects [88]. In order to investigate the true effect of jackfruit-derived extracts on colorectal tumorigenesis in vivo, we conducted animal experiments with AOM/DSS-induced colorectal cancer model.

2.2 Materials and methods

2.2.1 Animal experiment

C57BL/6 male mice at age 7 weeks, purchased from Charles River (Wilmington, MA), were maintained at the University of Massachusetts Amherst in a standard Specific Pathogen Free animal facility. The mice were divided into three groups: vehicle group had 11 mice, low dose jackfruit-derived fraction group (240 ppm) had 10 mice, high dose jackfruit-derived fraction group (480 ppm) had 10 mice. While mice of all groups were maintained on the same chow diet, all mice were injected with AOM (1mg/ml dissolved in PBS, 10mg/kg body weight); and one week later, the mice were given drinking water containing 2% DSS for one week. New diets were given to all groups of mice after one week of DSS treatment. Specifically, diets for all three groups consisted of 10 wt/wt % commercial corn oil and 0.5 mL/g % PEG 400 while the diet for low dose and high dose jackfruit-derived fraction groups also consisted of 240 ppm and 480 ppm jackfruit-derived fraction respectively. The profile of the composition of the prepared diets are listed in **Table 1**. At day 56 after the day when AOM was injected, the mice were sacrificed to collect colon tissue and plasma for analysis. For tumor analysis, colon tissues were cut open longitudinally and then washed with PBS. The washed tissues were inspected under a dissection microscope for quantification of colon tumor. The tumor size was calculated with the followed formula: tumor size = $\pi * (d/2)^2$ (d is the diameter of each tumor).

Table 1. Animal diet Composition

Ingredient (g/kg)	Vehicle group	Low dose jackfruit-derived fraction group	High dose jackfruit-derived fraction group
Casein	200	200	200
Cellulose	50	50	50
Choline Bitartrate	2.5	2.5	2.5
Cornstarch	367.5	367.5	367.5
Dyetrose	132	132	132
L-Cysine	3	3	3
Mineral Mix #210025	35	35	35
Sucrose	100	100	100
Vitamin Mix #310025	10	10	10
Commercial corn oil	100	100	100
PEG 400 (mL/kg)	5	5	5
Jackfruit-derived fraction	0	0.24	0.48

2.2.2 RT-PCR analysis of gene expression in colon tissues

For the analysis of gene expression, colon tissues from the same locations were frozen by liquid nitrogen and then got grounded. TRIzol reagent (Invitrogen, Carlsbad, CA) was added into the grounded samples to isolate total RNA from the colon tissues. The quality of the extracted RNA was measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the RNA was reverse transcript into cDNA with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. A DNA Engine Opticon system (Bio-Rad Laboratories, Hercules, CA) with Maxima SYBR-green Master Mix (Thermo Fisher Scientific) was used for the performance of RT-PCR. The sequences of mouse-specific primers (Thermo Fisher Scientific) used were listed as in **Table 2**. The

results of target genes were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) and expressed to the mice treated with control diets using the $2^{-\Delta\Delta C_t}$ method.

Table 2. Sequences of primers used in RT-PCR

Gene	Forward primer	Reverse primer
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
<i>Il-6</i>	TAGTCCTTCCTACCCCATTTCC	TTGGTCCTTAGCCACTCCTTC
<i>Ifn-γ</i>	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
<i>Axin2</i>	TGACTCTCCTTCCAGATCCCA	YGCCCACACTAGGCTGACA
<i>Vegf</i>	GCACATAGAGAGAATGAGCTTCC	CTCCGCTCTGAACAAGGCT
<i>Myc</i>	ATGCCCTCAACGTGAACTTC	GTCGCAGATGAAATAGGGCTG
<i>Pena</i>	TTTGAGGCACGCCTGATCC	GGAGACGTGAGACGAGTCCAT

2.2.3 Histological analysis of colon tissues

Parts of the dissected colon tissues were fixed in 4 % formalin (Thermo Fisher Scientific) for 48 hours. For H&E staining, the fixed tissues were embedded in paraffin (Thermo Fisher Scientific) and sliced by Rotary Microtome (Thermo Fisher Scientific) to 5 μm sections, and then dewaxed in serial xylene (Thermo Fisher Scientific) and rehydrated through graded ethanol solutions (Phamoc-Aaper, Brookfield, CT), stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO), examined with a light microscopy. To conduct immunohistochemistry analysis, the sections were heated in 0.01 M citrate buffer (pH 6.0) to 95 °C for 10 minutes to perform the antigen retrieval in a PT Module antigen retrieval device (Thermo Fisher Scientific). The samples were incubated with primary antibodies against PCNA and β-catenin (Cell Signaling Technology) at 4 °C overnight. The sections were applied with Horseradish peroxidase (HRP)-conjugated secondary antibodies, and then stained with chromogen 4-diaminobenzidine according to the instruction of HRP/DAB (ABC) Detection IHC kit (Abcam). The samples were then

counterstained for one minute with hematoxylin. Light microscope was latter used for observation of the positive expression of PCNA and β -catenin, and the quantification of the expression was done with ImageJ software.

2.2.4 Statistical analysis

All data were expressed as the mean \pm standard error of the mean (SEM). Statistical significance was determined by the Mann-Whitney test for the comparison between the vehicle and jackfruit-derived fraction treatment groups. The statistical analysis was performed with Prism 7.0 Version (Graphpad Software Inc., USA), and a *P* value less than 0.05 was used as the level suggesting statistical significance.

2.3 Results

2.3.1 Effect of jackfruit-derived fraction on body weight of mice

All groups of mice were injected with 1mg/ml AOM (dissolved in PBS) at week 0 and then stimulated with drinking water containing 2% DSS for seven days, in order to induce colon cancer, after two weeks' pre-treatment (see scheme of animal experiment in **Figure 1**). Body weight of mice were recorded every 7 days through the whole treatment process (**Figure 2**). New diets were given to all groups of mice after seven days of DSS treatment. Specifically, diets for all three groups consisted of 10 wt/wt % commercial corn oil and 0.5 mL/g % PEG 400 while the diet for low dose and high dose jackfruit-derived fraction groups also consisted of 240 ppm and 480 ppm jackfruit-derived fraction respectively. After the week treated with DSS in water, the vehicle group mice showed more stability in weight change than the mice treated with 480 ppm jackfruit-derived fraction diet and the mice treated with 240 ppm jackfruit-derived fraction group mice. Later during the treatment process, the mice treated with 240 ppm jackfruit-derived

fraction diet showed a tendency to gain more weight than the mice treated with vehicle diet and 480 ppm jackfruit-derived fraction diet, although the increase was not significant.

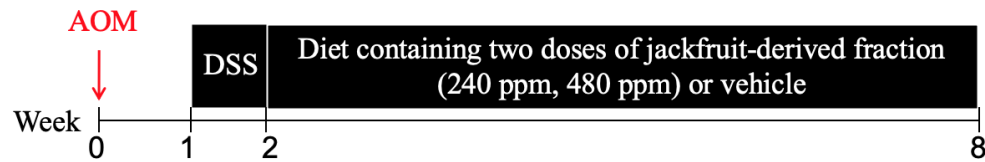


Figure 1: Animal experiment studying the effects of jackfruit-derived fraction (240 ppm, 480 ppm) on the AOM/DSS-induced colon tumor.

Scheme of animal experiment. AOM was injected to the three groups, followed with 2% DSS water treatment for 7 days. Mice then were given diet containing two doses of jackfruit-derived fraction (240 ppm, 480 ppm) or vehicle after DSS treatment for 42 days. The mice were sacrificed 56 days after AOM injection.

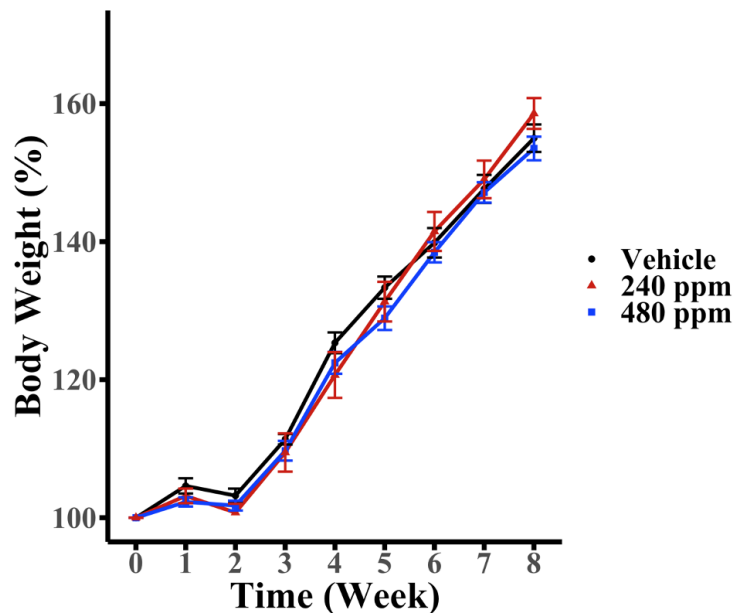


Figure 2: Effect of jackfruit-derived fraction (240 ppm, 480 ppm) on the body weight of mice.

Changes of body weight (expressed in percentage) for all groups of mice, since AOM injection. (n = 10-11 per group)

2.3.2 Jackfruit-derived fraction mitigates AOM/DSS-induced colon tumorigenesis *in vivo*

As shown by the data, the mice treated with 480 ppm jackfruit-derived fraction showed no significant change in total tumor size of the mice for vehicle group vs 480 ppm jackfruit-derived fraction group (**Figure 3B**, 33.77 ± 0.84 for vehicle group vs 2.35 ± 0.71 for 480 ppm jackfruit-derived fraction group, $P = 0.1188$, mean \pm SEM), while there are a significant decrease in tumor numbers of each mouse for vehicle group vs 480 ppm jackfruit-derived fraction group, up to 46% decrease compared with the mice treated with only vehicle (**Figure 3A**, 3.9 ± 0.67 for vehicle group vs 2.1 ± 0.31 for 480 ppm jackfruit-derived fraction group, $P = 0.0431$, mean \pm SEM), indicating alleviated colon tumorigenesis. Considering the lack of clear differences between vehicle group and 240 ppm jackfruit-derived fraction group in tumor numbers and in total tumor size of each mouse (**Figure 3A-B**), all following experiments are not conducted on 240 ppm jackfruit-derived fraction group.

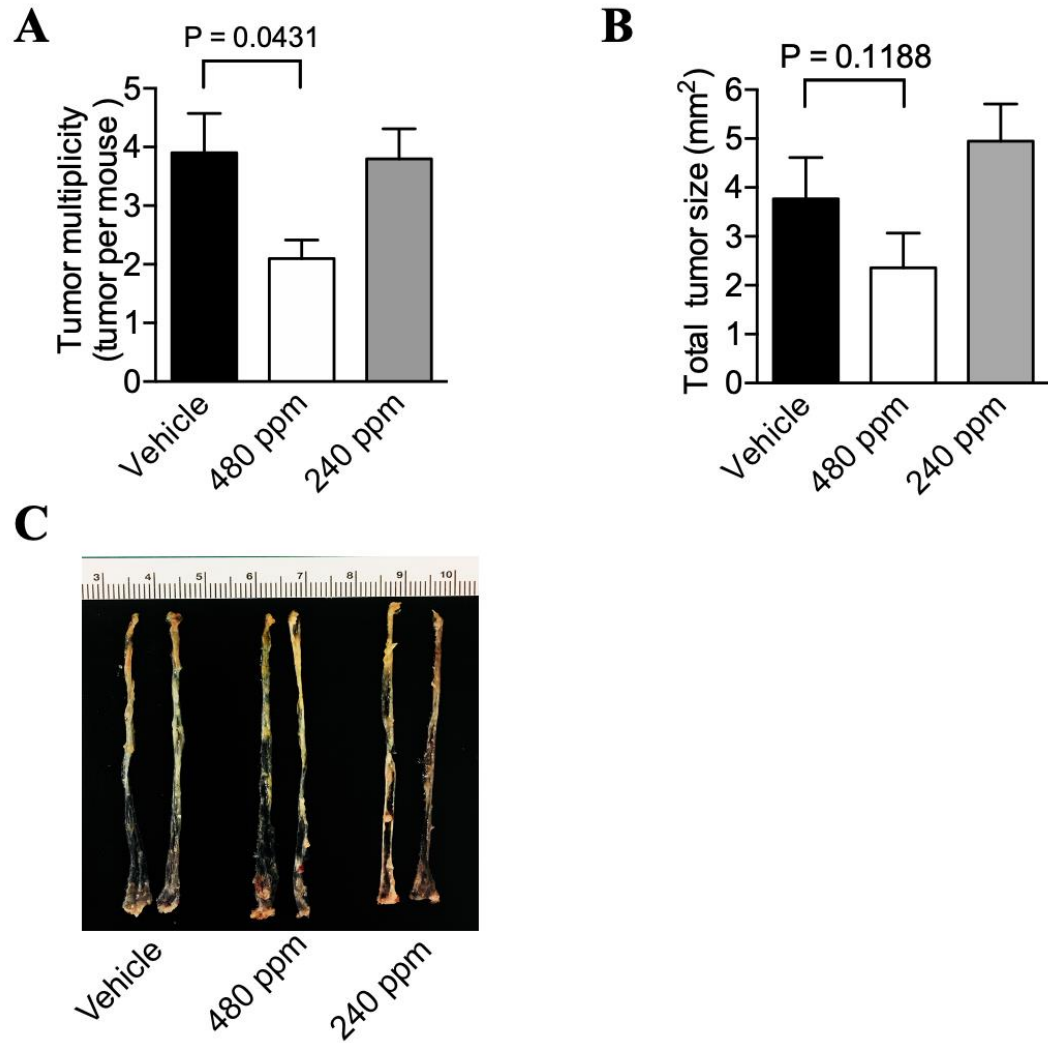


Figure 3: Compared with mice treated with only vehicle and 240 ppm jackfruit-derived fraction, AOM/DSS-induced colon tumorigenesis is decreased in 480 ppm jackfruit-derived fraction treated mice.

After sacrificing the mice on the 56th day since AOM injection, tumor in colon were counted and measured under a dissection microscope. (A-C) Quantification of colon tumorigenesis in all groups. The data are expressed by mean \pm SEM, and statistical significance is determined by Mann–Whitney *U* test, where *n* = 10–11 per group.

Consistent with the decreased tumorigenesis in colon, the pro-inflammatory cytokines (*Il-6* and *Inf- γ*) (**Figure 4 A-B**) and some pro-tumorigenic genes (*Axin2*, *Vegf*, *Myc* and *Pcna*) (**Figure 5 A-D**) were less expressed in the mice treated with 480 ppm jackfruit-derived fraction than the mice in vehicle group. H&E staining showed a decreased size of tumor in the colon of mice treated with 480 ppm jackfruit-derived fraction (**Figure 6 A**). Immunohistochemistry validated that 480 ppm jackfruit-derived fraction decreased the expression of proliferation cell nuclear antigen (PCNA), in colon tumors (**Figure 6 B**).

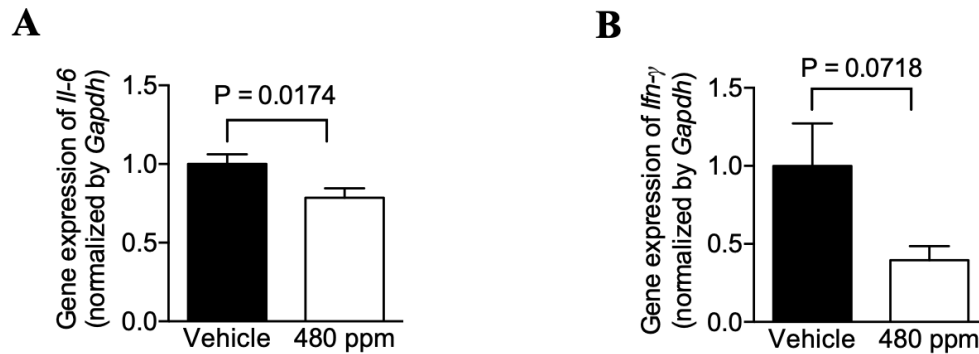


Figure 4: 480 ppm jackfruit-derived fraction consumption decreases the expression of pro-inflammatory cytokines *in vivo*.

Expression of pro-inflammatory genes, including (A) *Il-6*, (B) *Inf- γ* , in colon tissues. The data are expressed by mean \pm SEM, and statistical significance is determined by Mann–Whitney *U* test, where n = 10-11 per group.

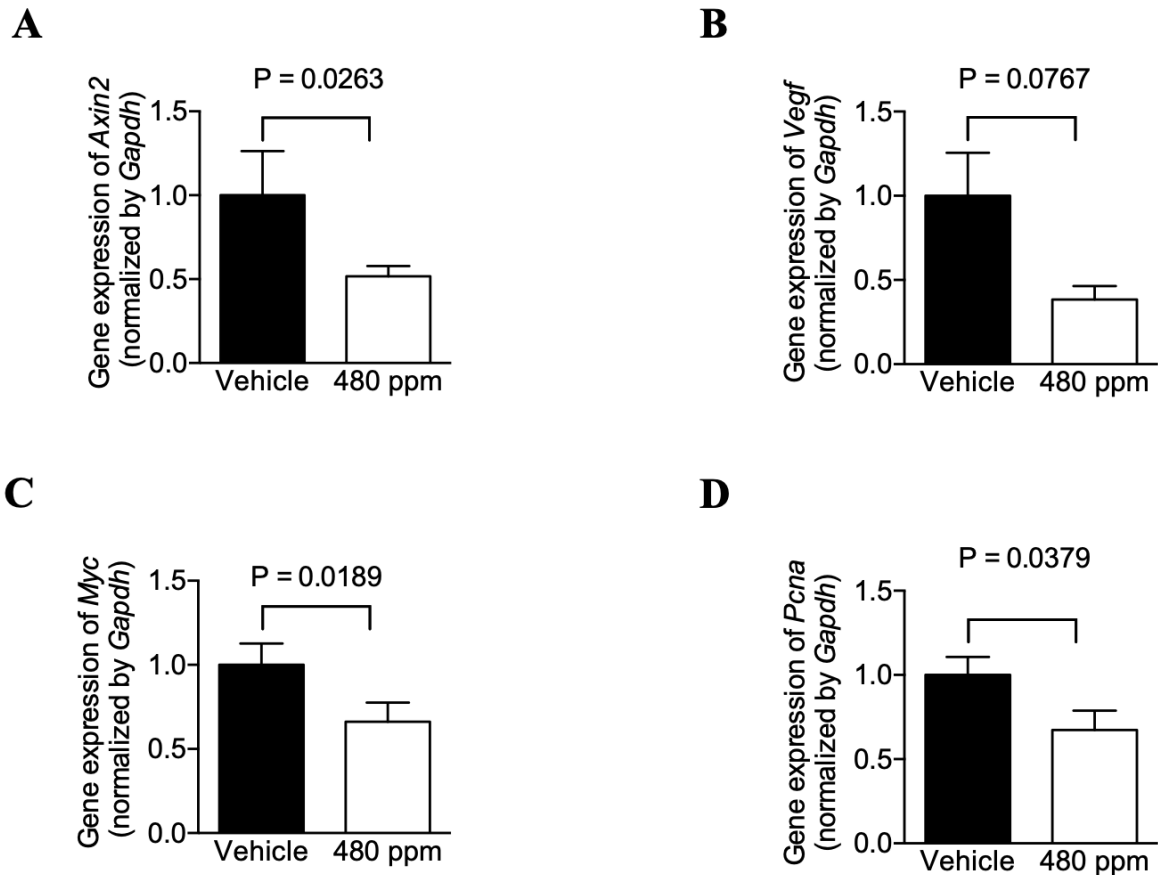


Figure 5: 480 ppm jackfruit-derived fraction consumption decreases the expression of pro-tumorigenic genes *in vivo*.

Expression of pro-tumorigenic genes, including (A) *Axin2*, (B) *Vegf*, (C) *Myc*, (D) *PcnA*, in colon tissues. The data are expressed by mean \pm SEM, and statistical significance is determined by Mann–Whitney *U* test, where $n = 10-11$ per group.

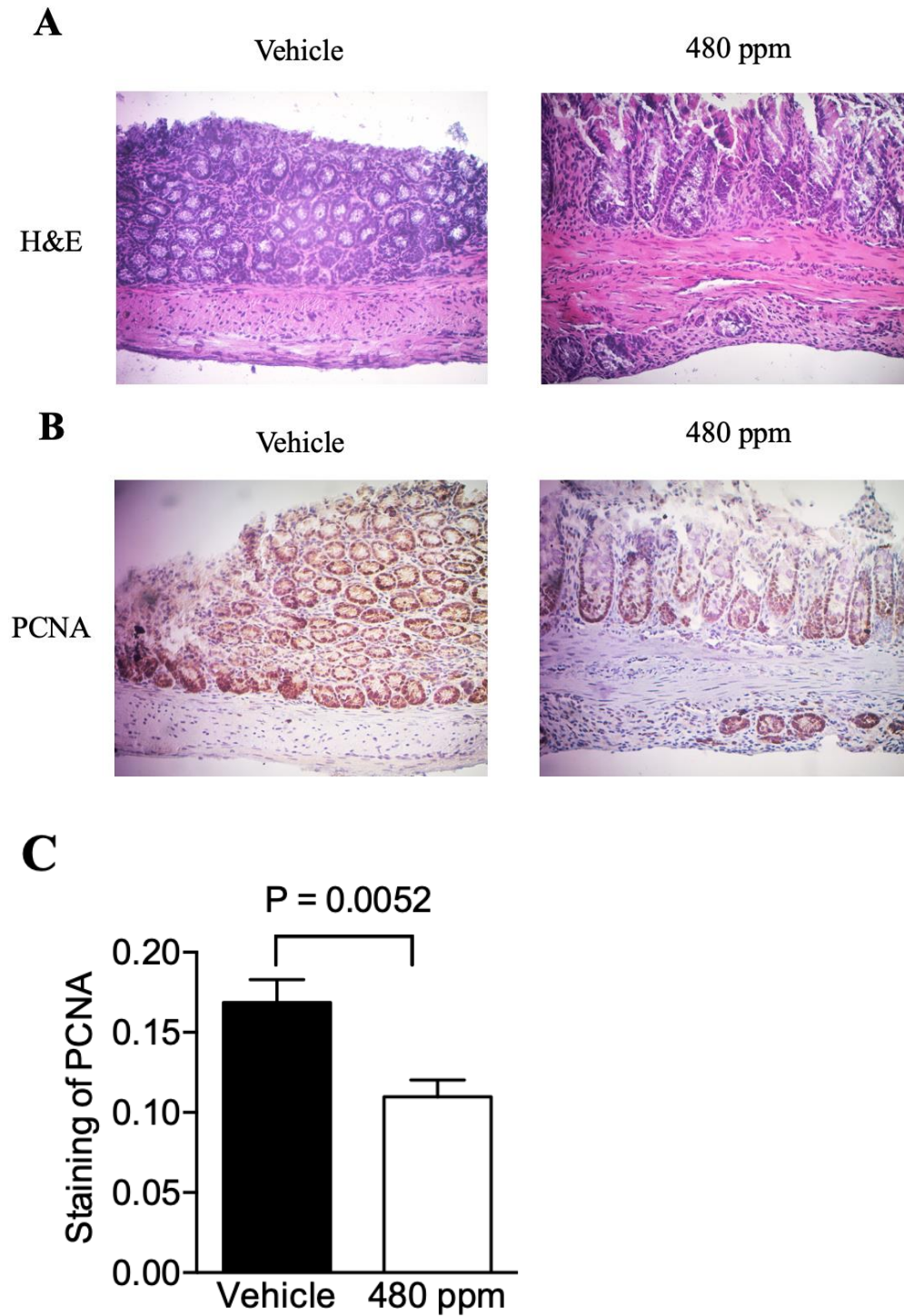


Figure 6: Immunohistochemical staining of H&E and PCNA.

The data are expressed by mean \pm SEM, and statistical significance is determined by Mann–Whitney *U* test, where $n = 10$ -11 per group.

2.4 Discussion

As the third most common cancer and the fourth most common cause of cancer-related death, with 700,000 deaths per year, exceeded only by lung, liver and stomach cancers, the incidence of colorectal cancer is increasing year by year, with 200,000 new cases per year from 1990 to 2012, calling more studies to be conducted on colorectal cancer prevention. Many studies have shown the potential of jackfruit, a common tropical fruit that is rich in carbohydrates, proteins, vitamins, minerals, and phytochemicals, in protecting against colon tumorigenesis *in vitro*. Our collaborators provided us the jackfruit-derived extract. Here, we conducted an AOM/DSS mice study on the potential chemopreventative effect of jackfruit-derived extracts on colon tumorigenesis.

We have found that the dietary consumption of 480 ppm jackfruit-derived extracts could mitigate the development of colon tumorigenesis in C57BL/6 male mice. We started the experiments with three groups: 240 ppm jackfruit-derived extracts, 480 ppm jackfruit-derived extracts and vehicle. However, no significant difference of the quantification of colon tumorigenesis was observed between 240 ppm jackfruit-derived extracts group and vehicle group. Therefore, further experiments were not conducted on 240 ppm jackfruit-derived extracts group.

CHAPTER 3

FUTURE WORK

Our experiments studied the effect of jackfruit-derived extracts on the development of colon tumorigenesis in C57BL/6 male mice. The analysis results all together supported that dietary consumption of high dose (480 ppm) jackfruit-derived extracts could alleviate colorectal tumorigenesis in mice. However, there are still some limits in the *in vivo* experiment. Therefore, much further work could be done in the future to investigate more in detail on the effect of this extract on colon tumorigenesis.

3.1 Dosage study

The quantification of colon tumorigenesis has shown that 240 ppm jackfruit-derived extract has no significant effect on alleviating colitis associated tumorigenesis in C57BL/6 male mice, possibly due to the lack of strong potency of natural products on the protection against carcinogenesis, when compared to synthetic cancer drugs. The fact that 240 ppm jackfruit-derived extract did not show significant effect suggests the effect might be dose-dependent. Thus, we can later set up more groups of different dosage to investigate the dose effect on the model.

3.2 Model study

Our experiment was done in only C57BL/6 male mice model. Such mice need an AOM/DSS treatment to initiate colitis-associated colorectal cancer. Considering the different causes of colorectal cancer among humans, other mouse models including genetically engineered mice could be used to investigate the effect of jackfruit on hereditary colorectal cancer including FAP and HNPCC for future studies.

3.3 Gender and age

We conducted the experiments only on male mice aged 7 weeks. However, it is reported that sex is also a factor in the risk of developing colorectal cancer, with 9.2% in women and 10% in men

By gender, CRC is the second most common cancer in women (9.2%) and the third in men (10%). A study conducted in 2008 has suggested that the main risk factor for colorectal cancer is age: past the fifth decade of life, the risk of developing CRC is markedly increased, while the onset of colorectal cancer below the age of fifty is rare (apart from inherited cancers) [89]. Both findings indicate the need for conducting *in vivo* experiments on the effect of jackfruit on colorectal cancer among different sex and age groups.

3.4 Composition study

The jackfruit-derived extract used in this experiment is believed to a crude extract containing many different compounds. It is difficult to understand that which one or which ones of the extract is playing a role on the development of colorectal cancer in the mice, calling for the need of isolating each compound, and assessing the potential of each compound on the effect of colorectal carcinogenesis. Moreover, we are unsure if any compositions of the extract mixture have changed from the time of diet preparation until the extract has been consumed. This suggests some stability studies of the extract.

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